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3. 20020160509 . 05 Mar 02. 31 Oct 02. Embryonic stem cells. Reubinoff, Benjamin Eithan, et al. 435/366; C12N005/08.

4. 20020068045 . 14 Mar 01. 06 Jun 02. Embryonic stem cells and neural progenitor cells derived therefrom. Reubinoff, Benjamin Eithan, et al. 424/93.7; 435/368 A61K045/00 C12N005/08.

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L3 5194 S NEURAL(3A)(PROGENITOR OR STEM)
L4 73352 S PLURIPOTENT OR ES OR EMBRYONIC(3A)(GERM OR STEM OR CARCINOMA)
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L7 115 S L6 AND L4
L8 88 S L6(S)L4
L9 45 DUP REM L8 (43 DUPLICATES REMOVED)

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L9 ANSWER 1 OF 45 BIOSIS COPYRIGHT 2002 BIOLOGICAL ABSTRACTS INC.
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AU Howard, M. J. (1); Chang, L. K. (1); Schottler, F. (1); St. Clair, A. M. (1); Adams, L. D. (1); Liu, S. (1); Stewart, T. J. (1); Holekamp, T. F. (1); Jacquin, M. F. (1); Gottlieb, D. I. (1); Johnson, E. M. (1); McDonald, J. W. (1)
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=> d ab 43-45 l9

L9 ANSWER 43 OF 45 MEDLINE DUPLICATE 22
AB During embryogenesis, **pluripotent** stem cells segregate into daughter lineages of progressively restricted developmental potential. In vitro, this process has been mimicked by the controlled differentiation of **embryonic stem** cells into **neural** precursors.
To explore the developmental potential of these cell-**culture**-derived precursors in vivo, we have implanted them into the ventricles of embryonic rats. The transplanted cells formed intraventricular neuroepithelial structures and migrated in large numbers into the brain tissue. **Embryonic-stem**-cell-derived neurons, astrocytes, and oligodendrocytes incorporated into telencephalic, diencephalic, and mesencephalic regions and assumed phenotypes indistinguishable from neighboring host cells. These observations indicate that entirely in vitro-generated neural precursors are able to respond to environmental signals guiding cell migration and differentiation and have the potential to reconstitute neuronal and glial lineages in the central nervous system.

L9 ANSWER 44 OF 45 MEDLINE DUPLICATE 23
AB Mutations at the Steel (Sl) and dominant white spotting (W) loci affect three **embryonic** lineages: primordial **germ** cells, hemopoietic **stem** cells and **neural**-crest-derived melanocytes. The gene products of these loci are a peptide growth factor, called here stem cell factor (SCF), and its tyrosine kinase receptor, the proto-oncogene c-kit. We have studied how chicken recombinant SCF affects the development of melanocytes from quail neural crest cells in secondary **culture** under defined conditions. We observed that the total number of neural crest cells, of melanocytes and of their precursors was higher in the presence than in the absence of SCF. Labelling with bromodeoxyuridine showed that SCF had a modest and transient mitogenic effect on the neural crest population. SCF also enhanced the differentiation rate of melanocyte precursors, recognized by the "melanocyte early marker" monoclonal antibody (MelEM MAb), and of melanocytes, since the proportion of both subpopulations significantly increased in the presence of SCF. Finally, SCF increased the survival of the neural crest population since in its presence the total number of cells remained stable while it gradually declined in control **cultures**. Our results support the notion that SCF sustains the survival of the neural crest population and stimulates the rate of the melanogenic differentiation process.

L9 ANSWER 45 OF 45 MEDLINE DUPLICATE 24
AB **Pluripotent** P19 embryonal carcinoma cell **cultures** can be induced to differentiate into neurons and glial cells by the addition of 10(-6) M retinoic acid. During early neural differentiation, a bundle of colchicine-stable, acetylated microtubules is formed. This acetylated microtubule array apparently extends to form neurites during neurogenesis. In this paper, we analyze changes in vimentin and MAP 2 distributions during neural differentiation with respect to the changes in the

acetylated microtubule array. During a brief period early in differentiation, indirect immunofluorescence staining shows the colocalization of colchicine-stable acetylated microtubules, vimentin, and MAP 2. Using acrylamide to disrupt the organization of vimentin intermediate filaments and estramustine to disrupt the binding of MAP 2 to microtubules, we show that acetylated microtubules, MAP 2, and vimentin intermediate filaments are arranged in an interdependent cytoskeletal array. We suggest this array may serve to stabilize processes in **neural stem** cells, before the final decision to differentiate into neurons or glia is made.

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AU Brustle O; Spiro A C; Karram K; Choudhary K; Okabe S; McKay R D
TI In vitro-generated neural precursors participate in mammalian brain development.
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1997 Dec 23) 94 (26) 14809-14.
Journal code: 7505876. ISSN: 0027-8424.

L9 ANSWER 44 OF 45 MEDLINE DUPLICATE 23
AU Lahav R; Lecoin L; Ziller C; Nataf V; Carnahan J F; Martin F H; Le Douarin N M
TI Effect of the Steel gene product on melanogenesis in avian neural crest cell cultures.
SO DIFFERENTIATION, (1994 Dec) 58 (2) 133-9.
Journal code: 0401650. ISSN: 0301-4681.

L9 ANSWER 45 OF 45 MEDLINE DUPLICATE 24
AU Falconer M M; Vielkind U; Brown D L
TI Association of acetylated microtubules, vimentin intermediate filaments, and MAP 2 during early neural differentiation in EC cell culture.
SO BIOCHEMISTRY AND CELL BIOLOGY, (1989 Sep) 67 (9) 537-44.
Journal code: 8606068. ISSN: 0829-8211.

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In vitro-generated neural precursors participate in mammalian brain development

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ABSTRACT **During embryogenesis, pluripotent stem cells segregate into daughter lineages of progressively restricted developmental potential. *In vitro*, this process has been mimicked by the controlled differentiation of embryonic stem cells into neural precursors. To explore the developmental potential of these cell-culture-derived precursors *in vivo*, we have implanted them into the ventricles of embryonic rats. The transplanted cells formed intraventricular neuroepithelial structures and migrated in large numbers into the brain tissue. Embryonic-stem-cell-derived neurons, astrocytes, and oligodendrocytes incorporated into telencephalic, diencephalic, and mesencephalic regions and assumed phenotypes indistinguishable from neighboring host cells. These observations indicate that entirely *in vitro*-generated neural precursors are able to respond to environmental signals guiding cell migration and differentiation and have the potential to reconstitute neuronal and glial lineages in the central nervous system.**

The ability to isolate, proliferate, and genetically manipulate embryonic stem (ES) cells is one of the major achievements in experimental biology (1, 2). The totipotency of these cells has kindled numerous efforts to generate tissue-specific precursors from ES cells *in vitro*. The controlled differentiation of ES cells into stem cells of defined lineages provides experimental access to early embryonic development and may eventually lead to alternative donor sources for tissue reconstruction. A central question concerning the biology of ES-cell-derived precursors is to what extent these cells resemble their *in vivo* counterparts. Are precursor cells generated outside the context of a multicellular organism sufficiently responsive to positional cues to participate in the development and histogenesis of a living host? Hematopoietic stem cells derived from ES cells have, indeed, been shown to reconstitute the lymphoid, myeloid, and erythroid lineages after transplantation into irradiated mice (3). During the last 3 years, several groups have demonstrated that mature neurons and glia can be derived from ES cells *in vitro* (4–7). Recently, we have reported an efficient procedure for the generation of proliferative neural precursors from ES cells. *In vitro*, these precursors generate functional neurons, astrocytes, and oligodendrocytes (8). *In vivo* reconstitution would be a more stringent test of the potential of ES cells to acquire central nervous system fates. The limited self-renewal in the adult mammalian brain precludes the kind of ablation and reconstitution experiments used to study *in vivo* differentiation of hematopoietic progenitors. We have, therefore, used a different approach and introduced ES-cell-derived neural precursors into the developing brain. Previous studies have revealed that primary neuroepithelial precursors implanted into the ventricle of embryonic rats incorporate extensively into the host brain and undergo region-specific differentiation into neurons and glia (9–11). Herein, we show that ES-cell-derived neural precursors grafted into the embryonic ventricle migrate into the host brain and contribute to all three principal lineages of the nervous system. Our results suggest that neuroepithelial precursors derived from ES cells in the absence of positional cues can migrate and differentiate according to local signals in the host environment.

MATERIALS AND METHODS

ES Cell Culture. ES cells (line J1; ref. 12) were maintained on γ-irradiated fibroblasts in DMEM containing 20% fetal calf serum, 0.1 mM 2-mercaptoethanol, nucleosides, nonessential amino acids, and human recombinant leukemia inhibitory factor (1000 units/ml). Cells were passaged once onto gelatin-coated dishes and then aggregated to form embryoid bodies in bacterial dishes in the absence of leukemia inhibitory factor. Four-day-old embryoid bodies were plated in tissue culture dishes and propagated for 5–12 days in ITSFn medium (DMEM/F-12 containing insulin at 5 μg/ml, transferrin at 50 μg/ml, 30 nM selenium chloride, and fibronectin at 5 μg/ml; ref. 8).

Intrauterine Transplantation. Cells were trypsinized and triturated to single-cell suspensions in the presence of 0.1% DNase. Timed-pregnant Sprague–Dawley rats were anesthetized with ketamine hydrochloride (80 mg/kg) and xylazine (10 mg/kg), and 0.1–1 × 10^6 cells were injected into the telencephalic vesicle of each embryo as described (11).

Immunohistochemistry. Zero to 15 days after spontaneous birth, recipients were anesthetized and perfused with 4% paraformaldhyde in PBS (stillborn recipients were fixed by immersion). Serial 50-μm Vibratome sections were characterized with antibodies to microtubule-associated protein 2 (Sigma, dilution, 1:200), nestin (dilution, 1:1,000), glial fibrillary acidic protein (GFAP; ICN, dilution, 1:100), (CNPase; Sigma, dilution, 1:200), neurofilament (SMI311, Sternberger Monoclonals, Baltimore, MD; dilution, 1:500), NeuN (Chemicon, dilution, 1:50), tyrosine hydroxylase (Eugene Tech Intl., Ridgefield Park, NJ; dilution, 1:200), M2 and M6 (refs. 13 and 14; dilution, 1:10). Antigens were visualized by using appropriate fluorophore- or peroxidase-conjugated secondary antibodies. To assay alkaline phosphatase activity, sections were incubated at 37°C in 100 mM Tris·HCl, pH 9.5/100 mM

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Abbreviations: E, embryonic day; ES cell, embryonic stem cell; P, postnatal day; GFAP, glial fibrillary acidic protein; CNPase, 2′,3′-cyclic nucleotide 3′-phosphodiesterase.

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NaCl/50 mM $MgCl_2$/nitroblue tetrazolium at 0.3 mg/ml/5-bromo-4-chloro-3-indolyl phosphate at 0.175 mg/ml. After 3–10 min, the staining was stopped by transferring the sections to 10 mM Tris·HCl/1 mM EDTA. Specimens were examined on Zeiss Axioplan, Axiovert, and Laser Scan microscopes.

***In Situ* Hybridization.** Donor cells were identified by using a digoxigenin-end-labeled oligonucleotide probe to the mouse major satellite (15). DNA–DNA *in situ* hybridization was performed as described (11). Briefly, sections were treated with Pronase at 25 μg/ml in 2× SSC/5 mM EDTA for 15 min at 37°C, dehydrated, and denatured in 70% formamide/2× SSC at 85°C for 12 min. After dehydration in ice-cold ethanols, sections were hybridized overnight at 37°C in 65% formamide/2× SSC/salmon sperm DNA at 250 μg/ml. Washes were 50% formamide/2× SSC (30 min, 37°C) and 0.5× SSC (37°C, 15 min). Hybridized probe was detected by using alkaline phosphatase or fluorophore-conjugated antibodies to digoxigenin (Boehringer).

RESULTS

Widespread Incorporation of ES-Cell-Derived Neural Precursors into the Developing Brain. For transplantation, 4-day embryoid bodies were plated on tissue culture dishes and grown in ITSFn medium. This medium has previously been shown to strongly select for neural precursors (8). During the first 72 h in ITSFn, a large proportion of the cells died. Most of the remaining cells acquired an elongated phenotype strongly reminiscent of neuroepithelial precursor cells. These cells also expressed nestin, an intermediate filament typically present in neural precursor cells (16). After 6 days in ITSFn medium, typically more than 80% of the cells were nestin-positive. The remaining cells showed varied morphologies with focal expression of SSEA-1 and keratin 8, i.e., antigens typically expressed in undifferentiated embryonic tissues and primitive ectoderm (refs. 17 and 18; data not shown). After 5–12 days in ITSFn, cells were harvested and used for transplantation. Recipient animals were grafted between embryonic day (E) 16 and E18 and sacrificed between postnatal day (P) 0 and P15. Clusters of donor cells were detected in the ventricles of all successfully injected pups (see below). *In situ* hybridization revealed that large numbers of mouse cells left the ventricle and migrated into various host brain regions, including cortex, striatum, septum, thalamus, hypothalamus, and tectum (Figs. 1 and 2 *A*, *E*, *F*, and *K* and Table 1). The transplanted cells integrated individually into the host tissue and were only detectable by virtue of their genetic difference. The number of incorporated cells varied considerably among individual recipients and brain regions. Quantitative stereology and a detailed assessment of donor cell survival and proliferation will be required to assess what proportion of the transplanted cells integrates into the host brain. Preliminary cell counts have revealed up to 650 incorporated cells in single coronal 50-μm sections.

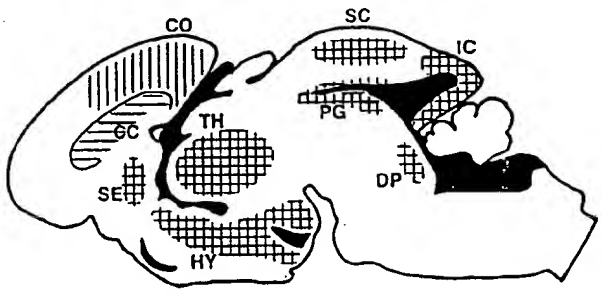

FIG. 1. Distribution of ES-cell-derived neural precursors after implantation into the telencephalic vesicle of E16–E18 rats. The schematic represents a midsagittal section through the brain of a newborn recipient. After leaving the ventricular system (solid areas), neurons (vertical lines) and astrocytes (horizontal lines) occupy overlying territories. Donor-derived neurons integrate preferentially into gray matter regions exhibiting neurogenesis until or beyond the time of implantation. ES-cell-derived astrocytes also incorporate into white matter regions such as the corpus callosum (CC). CO, cortex; DP, dorsal pontine area; HY, hypothalamus; IC, inferior colliculus; PG, periaqueductal gray; SC, superior colliculus; SE, septum; TH, thalamus. Donor-derived neurons and astrocytes were also detected in hippocampus (Fig. 3*B*), olfactory bulb (Fig. 4 *A* and *B*), and striatum (Fig. 2*E*).

Differentiation into Neurons and Glia. The differentiation of the incorporated cells was assessed by using antibodies to cell-type-specific antigens in conjunction with a mouse-specific DNA probe or mouse-specific antibodies to M6 and M2 (13, 14). Hybridized neurons expressing the neuronal antigens NeuN (19) and microtubule-associated protein 2 were detected at tel-, di-, and mesencephalic levels (Fig. 2 *I* and *L*). The shape, size, and orientation of these cells were indistinguishable from adjacent host neurons (Fig. 2*I*). Confocal laser microscopy allowed detailed reconstruction of individual phenotypes. ES-cell-derived neurons exhibited characteristic polar morphologies with segregation of neurites into dendrites and axons (Fig. 2 *B–D* and *G*). Both classes of neurites frequently extended several hundred micrometers into the adjacent host neuropil (Fig. 2 *G* and *H*). Donor-derived neurons were readily detectable at birth but appeared to undergo further morphological maturation in the postnatal period. The example in Fig. 2*M* shows an ES-cell-derived neuron with prominent dendritic spines in the thalamus of a 2-week-old host. Neurons integrating into the host cortex frequently displayed morphologies of projection neurons with long apical dendrites reaching into the superficial cortical layers and basal axons extending several hundred micrometers into the corpus callosum (Fig. 2 *B–D*). An example of a ES-cell-derived pyramidal neuron in the cortex of a neonatal host is shown in Fig. 2*D*. The donor neurons generated an extensive axonal network throughout the host gray and white matter, reaching from the most rostral regions such as the olfactory bulb to the brainstem (Fig. 3). Within the white matter, donor-derived axons frequently assembled into prominent fiber bundles running alongside host axons through the major axonal trajectories, including the corpus callosum (Fig. 3*A*), anterior commissure, striatal fiber bundles (Fig. 3*C*), and various other endogenous fiber tracts. Gray matter regions exhibiting dense donor-derived axonal networks included cortex (Fig. 3*A*), hippocampus (Fig. 3*B*), septum, striatum, thalamus (Fig. 3*D*), hypothalamus, tegmentum, tectum, and brainstem.

Astrocytes generated by the transplanted ES cells were found in a distribution similar to that of donor-derived neurons. The most prominent accumulations were detected in the ventral diencephalon and in tectum. In addition, these cells efficiently incorporated into white matter regions such as the corpus callosum (Fig. 1). Donor-derived astrocytes strongly expressed M2, a species-specific antigen frequently used for the identification of mouse astrocytes in xenografts (Fig. 4 *A* and *B* and ref. 21). Their astroglial identity was confirmed by double labeling with an antibody to GFAP of cells labeled with either the mouse satellite probe or the M2 antibody (Fig. 4*B*). Encountered only occasionally in newborn recipients, these cells were more frequently detected in animals sacrificed at P15 (Table 1). ES-cell-derived astrocytes were morphologically indistinguishable from their host counterparts, and GFAP immunofluorescence showed no differences in size or branching pattern between the two populations. Typical for astroglia, donor-derived astrocytes often extended processes to adjacent blood vessels.

In addition to neurons and astrocytes, donor-derived oligodendrocytes were found in the transplanted rat brains. The identity of these cells was confirmed by *in situ* hybridization with the mouse satellite probe and subsequent immunohisto-

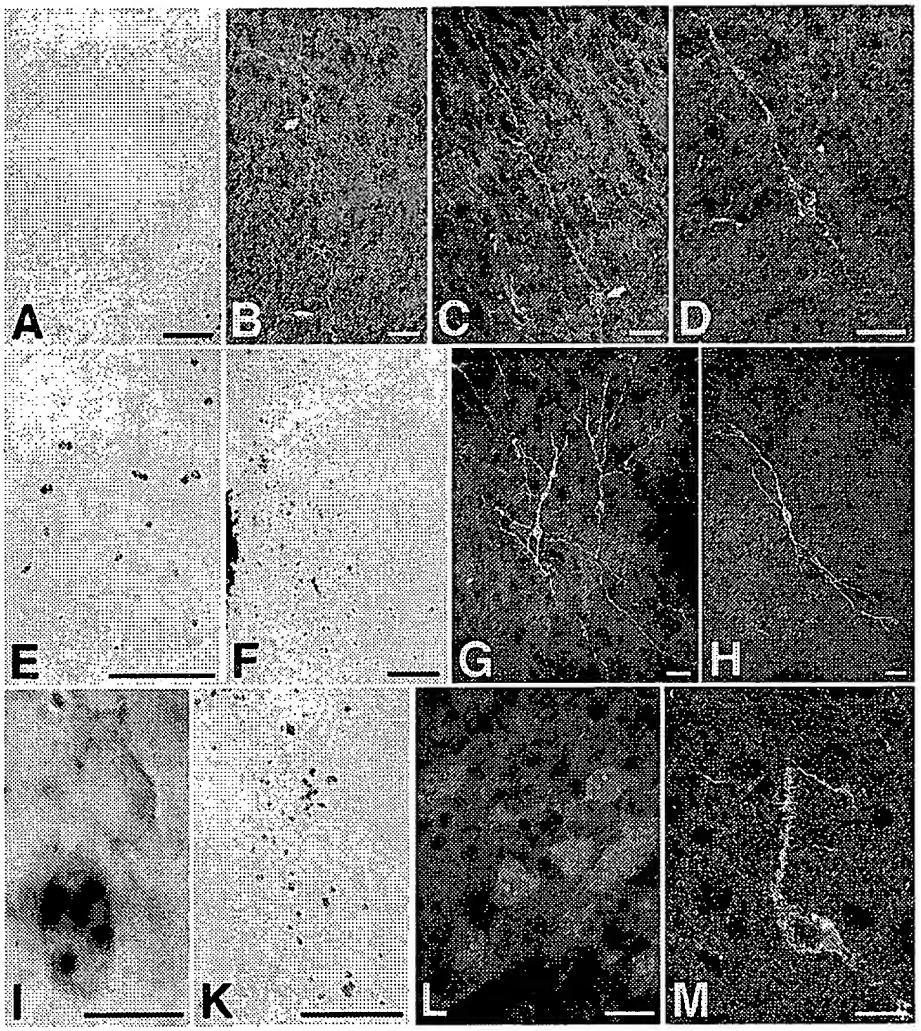

FIG. 2. ES-cell-derived neural precursors injected into the telencephalic vesicle of fetal rats incorporate individually into a variety of host brain regions and differentiate into neurons. Donor cells are identified by *in situ* hybridization using a digoxigenin-labeled probe to mouse satellite DNA (*A, E, F,* and *I–L*). Immunofluorescence detection of the mouse-specific antigen M6 and confocal laser microscopy were used to reconstruct individual neuronal profiles (*B–D, G, H,* and *M*). (*A–D*) Six days after injection into the telencephalic vesicle of an E17 rat, donor cells have left the ventricle and incorporated into the host cortex. The ES-cell-derived neurons show prominent apical dendrites and basal axons entering the corpus callosum, a morphology appropriate for cortical projection neurons (arrows: perikaryon). Note the characteristic pyramidal morphology in *D*. (*E*) Incorporated donor cells in the striatum of a 2-week-old rat transplanted at E18. (*F–H*) Donor-derived cells in the host hypothalamus. In contrast to cortex, neurons incorporating into the diencephalon frequently exhibited multipolar morphologies (*G* and *H*). (*I*) Host- and donor-derived neurons in the septum of a newborn rat. Both neurons show expression of microtubule-associated protein 2; the donor-derived cell is identified by *in situ* hybridization. (*K–M*) Incorporated cells in the host thalamus of newborn (*K*) and 2-week-old rats (*L* and *M*). In *L*, ES-cell-derived neurons are visualized by fluorescence *in situ* hybridization (green dots) and subsequent immunofluorescence analysis with an antibody to the nuclear neuronal antigen NeuN (red). Note the mature neuronal phenotype of the integrated cells with the presence of dendritic spines (*M*). [Bars = 100 μm (*A, E, F,* and *K*), 20 μm (*B–D, G, H, L,* and *M*), and 10 μm (*I*).]

chemical detection of CNPase, a marker for myelin and oligodendroglia (22). ES-cell-derived CNPase-positive oligodendrocytes were only detected in the brains of 2-week survivors and their distribution was restricted to white matter regions such as the corpus callosum and striatal fiber tracts. Size, orientation, and CNPase expression of these cells were indistinguishable from their host counterparts (Fig. 4 *C* and *D*).

Formation of Mitotic Neuroepithelium. Cells remaining in the host ventricle formed large clusters containing prominent neuroepithelial formations. These formations were particularly prominent in the 2-week survivors and—upon cross-sectioning—closely resembled tightly packed neural tubes (Fig. 5*A*). Individual tubes consisted of columnar epithelium with high mitotic activity at the luminal surface (Fig. 5*A Inset*). The epithelial cells showed strong expression of nestin (Fig. 5 *B* and *C*) and brain fatty-acid-binding protein (data not shown), antigens typically expressed in the early neuroepithelium (16, 23, 24). Cells expressing neuronal antigens, including microtubule-associated protein 2, neurofilament, and tyrosine hydroxylase, were restricted to the periphery of the formations, suggesting an inside-out gradient of differentiation (Fig. 5*D*). Thus, by morphology, antigen expression, and gradients of proliferation and differentiation, these structures are very similar to the developing neuroepithelium. In addition, intraventricular grafts contained small clusters of still undifferentiated embryonic cells. These cells were easily recognized by their expression of alkaline phosphatase, an enzyme typically present in undifferentiated embryonic cells (25). Whereas clusters of alkaline phosphatase-positive cells were frequently

Table 1. Differentiation of ES-cell-derived neural precursors after transplantation into the embryonic rat brain

	Parenchyma		Ventricle		
Age at analysis	Neurons	Glia	NEF	AP	NNT
P0	TDM	D	+	+	−
P0	TDM	T	−	+	−
P0	TD	−	+	−	−
P0	TM	−	−	+	−
P0	TDM	T	−	++	−
P0	D	D	+	+	−
P1	TDM	−	−	++	−
P1	TD	−	−	++	−
P1	TDM	−	−	+	−
P1	TD	−	−	+	−
P1	TDM	TDM	−	+	−
P1	TDM	−	−	+	−
P1	TDM	D	−	+	−
P1	TDM	TM	−	++	−
P15	DM	DM	+	+	+
P15	TDM	TD	−	−	+
P15	TDM	TDM	+	−	+
P15	TM	TM	+	+	+
P15	T	TD	+	−	+
P15	TDM	TM	+	−	+
P15	TD	TD	+	+	+

ES cells (line J1) aggregated to embryoid bodies and grown for 5–12 days in ITSFn medium were injected into the ventricle of E16–E18 rats. Recipients were sacrificed between P0 and P15 and donor-derived neurons were identified by DNA *in situ* hybridization in conjunction with immunohistochemical detection of NeuN or by expression of M6 and unequivocal morphological criteria (presence of dendrites and axons). ES-cell-derived astrocytes were detected with an antibody to M2 or by DNA *in situ* hybridization in conjunction with immunohistochemical detection of GFAP. Intraventricular donor cell clusters were assayed for the presence of nestin-positive neuroepithelial formations (NEF), clusters of undifferentiated, alkaline phosphatase-positive cells (AP; ++, numerous clusters; +, occasional clusters), and differentiated nonneural tissue (NNT). Each row represents one recipient animal. The integration patterns show considerable interindividual variability. There is an increase of glial cells and a decrease of AP-positive cells with increasing survival time. T, telencephalon; D, diencephalon; M, mesencephalon.

encountered in newborn animals, they were only occasionally detected in the 2-week survivors, arguing for a gradual differentiation of the embryonic cells. Disappearance of the alkaline phosphatase-positive cells was inversely correlated with the emergence of nonneural tissue in the P15 animals, where occasional islands containing adenoid structures, cartilage, and epidermis were found within the intraventricular clusters (Table 1). These observations indicate that undifferentiated embryonic cells present in the transplanted cell suspension form intraventricular teratomas.

DISCUSSION

Our results demonstrate that ES-cell-derived neural precursors transplanted into the developing mammalian brain are able to generate neuronal and glial lineages. Upon injection into the telencephalic vesicle of fetal rats, the donor cells leave the ventricle and migrate into the recipient brain, where they differentiate into neurons, astrocytes, and oligodendrocytes. Incorporation of the transplanted cells is not random but occurs in patterns compatible with the host brain development. The donor cells integrated preferentially into cortex, hippocampus, striatum, septum, the medial diencephalic nuclei, and tectum. These regions are known to continue neurogenesis until or even beyond the time of transplantation (26). Temporally, neurons, astrocytes, and oligodendrocytes appeared successively. Few astrocytes were present neonatally when

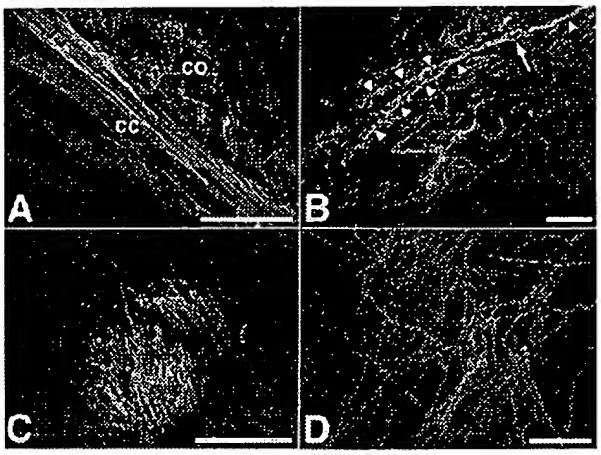

FIG. 3. Extensive axonal innervation of the host brain. The ES-cell-derived neurons generated a dense axonal network within the recipient brains. Abundant M6-positive axons were found at all levels in both gray and white matter. (*A*) Donor-derived axons in corpus callosum (cc) and deep layer cortex (co) of a 2-week-old recipient. (*B*) Axonal innervation of the hippocampal stratum oriens. The M6 immunofluorescence also depicts the perikaryon (arrow) and dendrites (arrowheads) of a large horizontal neuron in the upper stratum oriens. The morphology of this cell is very similar to the outline of Golgi-impregnated horizontal neurons in this area (20). (*C*) Abundant donor-derived axons in a striatal fiber tract of 2-week-old recipient brain. (*D*) ES-cell-derived axons in the thalamus of a newborn recipient transplanted at E17. (Bars = 50 μm.)

compared with 2-week-old recipients (Table 1), and oligodendrocytes were only detected in the P15 animals. This delayed appearance of glial cells corresponds well with the timing of host gliogenesis that has been shown to be primarily a postnatal event (27). In addition, neurons and glia showed differences in their distribution. Although neurons incorporated preferentially into gray matter regions, astrocytes invaded both gray and white matter, and oligodendrocytes were found only in fiber tracts. These observations indicate that neural precursors

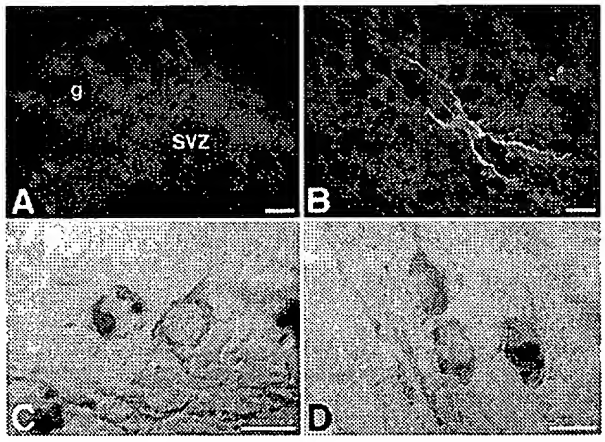

FIG. 4. Incorporation of ES-cell-derived glia. (*A* and *B*) ES-cell-derived astrocytes have migrated into the granular layer (g) of the olfactory bulb of a 2-week-old host. Cells are visualized with an antibody to the mouse-specific antigen M2 (red). SVZ, olfactory subventricular zone. An individual astrocyte, double labeled with an antibody to GFAP (green), is shown in *B*. (*C* and *D*) ES-cell-derived oligodendrocytes in the rostral (*C*) and caudal (*D*) corpus callosum of a 2-week-old host brain. The donor cells, identified by DNA *in situ* hybridization (black), are morphologically indistinguishable from adjacent host oligodendrocytes. Host- and donor-derived oligodendrocytes exhibit equivalent immunoreactivity to CNPase (red). [Bars = 100 μm (*A*) and 10 μm (*B–D*).]

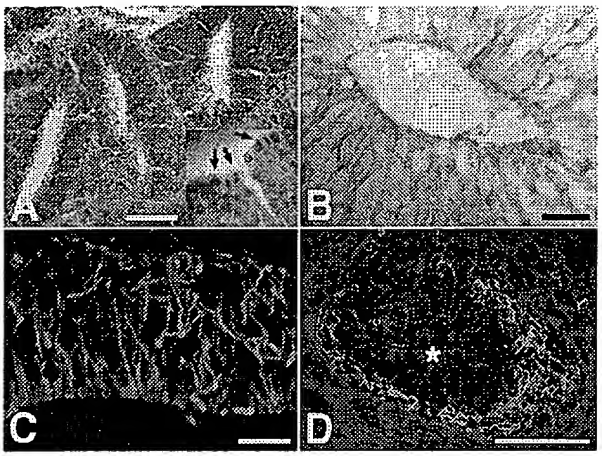

FIG. 5. Generation of neuroepithelial formations. (*A* and *B*) Eight days after intrauterine transplantation, the donor cells have generated numerous neural tube-like structures within the host ventricle. Like the developing neural tube, these structures exhibit high mitotic activity at the luminal surface (*A*) (hematoxylin/eosin; arrows in *Inset* indicate mitotic figures) and strong expression of the intermediate filament nestin (*B*). (*C* and *D*) Neuroepithelial formation in the ventricle of a 2-week-old animal transplanted at E18. The formation contains abundant radially oriented nestin-positive processes (*C*). As in the early neuroepithelium, there is an inside-out gradient of differentiation with neuronal markers being expressed at the periphery of the formation (*D*) (green, tyrosine hydroxylase; red, M6; *, center of formation). [Bars = 100 μm (*A* and *D*) and 20 μm (*B* and *C*).]

derived from ES cells and transplanted into the mammalian brain are susceptible to environmental cues guiding cell fate determination and differential migration. Furthermore, donor-derived neurons appear to respond to local differentiation cues. This is particularly evident in cortex, where they acquired morphological features characteristic for cortical projection neurons, including a pyramidal cell body, long apical dendrites, and basal axons projecting into the corpus callosum (Fig. 2 *B–D*). The innervation of the host brain by the transplanted cells further suggests that donor-derived neurons remain responsive to host-mediated axon guidance. Detectable at all levels of the recipient brain, donor-derived axons bundled and extended through the major host trajectories, running alongside endogenous axons and obeying the border of the fiber tracts (Fig. 3*C*). Donor-derived astrocytes and oligodendrocytes were similarly indistinguishable from their endogenous counterparts and only detectable by virtue of their genetic difference. Donor and host glia exhibited equivalent expression of the cell-type-specific antigens GFAP and CNPase. Many of the ES-cell-derived astrocytes assumed perivascular locations, with one or several processes extending to the capillary wall—a feature typically observed in astrocytes involved in blood–brain barrier formation (data not shown).

The migration and differentiation patterns of the transplanted ES cells are in accordance with those obtained after intrauterine transplantation of primary cells derived from the embryonic brain (9–11). In both cases, the donor cells incorporate preferentially into regions exhibiting protracted neurogenesis until late gestation and adopt local phenotypic features. These observations indicate that primary neuroepithelial cells and neural precursors derived from ES cells respond very similarly to environmental cues. Our data strongly suggest that neuroepithelial cells generated *in vitro* from ES cells can act as neural precursors *in vivo* and contribute neurons and glia to the developing mammalian brain. These findings significantly extend results of previous studies showing that retinoic acid-induced ES cells exhibit antigenic and electrophysiological properties of neurons and glia *in vitro* (4–7) and that the differentiated phenotype of retinoic acid-induced ES or teratocarcinoma cells can be maintained after transplantation into the adult brain (28–32). In contrast to these studies, we were less interested in the forceful induction and subsequent maintenance of a neuronal phenotype but rather in the interaction of still undifferentiated neural precursors with the developing brain. The widespread neuronal and glial integration obtained after transplantation of ES-cell-derived precursors provides an impressive example for the dominant role of non-cell-autonomous signals during neural migration and differentiation. The fact that a cell never previously exposed to a nervous system is able to migrate into cortex and to differentiate into an appropriate local phenotype illustrates that cell communities in individual brain regions harbor sufficient cues to maintain their local identity and to foster their own development through precursor cell recruitment.

It is a well-described phenomenon that ES cells transplanted to an adult host frequently develop teratomas and teratocarcinomas (for review, see ref. 33). In striking contrast, both ES and teratocarcinoma cells have been shown to participate in normal development upon introduction into early embryos at the blastocyst stage (for review, see ref. 34). Our observations, along with similar studies on ES-cell-derived hematopoietic progenitors (3), provide an interesting intermediate between these two scenarios. They show that the ability to participate in normal development is not restricted to undifferentiated ES cells but extends to their more differentiated progeny. Neural and hematopoietic precursors derived from ES cells *in vitro* are able to reconstitute neural and hematopoietic lineages after transplantation into living hosts. It is likely that such a contribution to host tissue formation requires close physical contact between the transplanted cells and the target tissue. One explanation for the conspicuous formation of neural tube-like structures within the host ventricles might be that ES cells differentiated into neural precursors but physically separated from the brain tissue are not sufficiently exposed to local cues mediating precursor cell recruitment and thus develop autonomously into primitive nervous system tissue. ES cells not sufficiently differentiated into neural precursors might evade recruitment because they are unable to respond to tissue-specific guidance cues. Because of their pluripotency, these cells can develop into a variety of tissues. Islands of nonneural tissue observed within the intraventricular clusters are most likely derivatives of still undifferentiated ES cells present in the transplanted cell suspension.

Although our results outline the developmental potential of *in vitro*-generated neural precursors at a basic neurobiological level, the *in vivo* reconstitution of neuronal and glial lineages by transplanted ES-cell-derived donor cells might eventually be exploited for cell replacement strategies. This idea receives strong impetus from recent findings implying that embryonic stem cells can be obtained from adult tissue by transferring nuclei of differentiated cells into oocytes (35), a perspective offering the possibility to generate virtually unlimited numbers of tissue-specific and genetically modified donor cells from the same individual. Transplant experiments in rodents further indicate that the adult brain may retain some of the cues required for regional cellular differentiation (36–38). These observations would suggest that region-specific differentiation of transplanted ES-cell-derived precursors is not limited to the developing nervous system. The feasibility of ES-cell-based replacement strategies will critically depend on the ability to generate highly purified donor populations susceptible to host regulation.

The possibility of introducing ES-cell-derived neurons and glia into the developing nervous system also offers an exciting approach for the study of neurological mutants. The rapid proliferation of ES cells and their susceptibility to genetic

manipulation allows the generation of large numbers of genetically modified donor cells. The properties of these cells can then be assayed *in vivo* in a wild-type recipient brain. The data presented herein show that the full range of neuronal and glial phenotypes might be accessible with this approach. Analysis is not restricted to cell migration and differentiation but may include aspects such as axon outgrowth and guidance, myelination, and the susceptibility of distinct genotypes to degenerative and neoplastic brain disease. Transplantation of ES-cell-derived neural precursors should be especially useful for the analysis of targeted gene deletions. Homozygous knockout ES cells from mutants exhibiting very early embryonic lethality can be differentiated *in vitro* and their neural offspring can be analyzed in the context of a developing brain. Incorporation of ES-cell-derived precursors into the central nervous system also offers the possibility to analyze the neural phenotype of null mutants without prior generation of a knockout animal.

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TI In vitro-generated neural precursors participate in mammalian brain development.
SO PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1997 Dec 23) 94 (26) 14809-14.
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Journal code: 8606068. ISSN: 0829-8211.

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Association of acetylated micr tubules, vimentin intermediate filaments, and MAP 2 during early neural differentiation in EC cell culture

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FALCONER, M. M., VIELKIND, U., and BROWN, D. L. 1989. Association of acetylated microtubules, vimentin intermediate filaments, and MAP 2 during early neural differentiation in EC cell culture. Biochem. Cell Biol. 67: 537–544.

Pluripotent P19 embryonal carcinoma cell cultures can be induced to differentiate into neurons and glial cells by the addition of 10^{-6} M retinoic acid. During early neural differentiation, a bundle of colchicine-stable, acetylated microtubules is formed. This acetylated microtubule array apparently extends to form neurites during neurogenesis. In this paper, we analyze changes in vimentin and MAP 2 distributions during neural differentiation with respect to the changes in the acetylated microtubule array. During a brief period early in differentiation, indirect immunofluorescence staining shows the colocalization of colchicine-stable acetylated microtubules, vimentin, and MAP 2. Using acrylamide to disrupt the organization of vimentin intermediate filaments and estramustine to disrupt the binding of MAP 2 to microtubules, we show that acetylated microtubules, MAP 2, and vimentin intermediate filaments are arranged in an interdependent cytoskeletal array. We suggest this array may serve to stabilize processes in neural stem cells, before the final decision to differentiate into neurons or glia is made.

Key words: embryonal carcinoma culture, MAP 2, microtubules, neural differentiation, vimentin.

FALCONER, M. M., VIELKIND, U., et BROWN, D. L. 1989. Association of acetylated microtubules, vimentin intermediate filaments, and MAP 2 during early neural differentiation in EC cell culture. Biochem. Cell Biol. 67 : 537–544.

Les cellules du carcinome embryonnaire pluripotentiel P19 en culture peuvent être induites à se différencier en neurones et en cellules gliales par addition d'acide rétinoïque 10^{-6} M. Au début de la différenciation neurale, il se forme un faisceau de microtubules acétylés, stables à la colchicine. Ce jeu de microtubules acétylés semble s'étendre pour former des neurites durant la neurogenèse. Nous analysons ici les changements dans la distribution de la vimentine et de la MAP 2 durant la différenciation neurale par rapport aux changements dans le jeu des microtubules acétylés. Durant une brève période du début de la différenciation, la coloration par immunofluorescence montre la colocalisation des microtubules acétylés stables à la colchicine, de la vimentine et de la MAP 2. Utilisant l'acrylamide pour briser l'organisation des filaments intermédiaires de vimentine et l'estramustine pour rompre la liaison de la MAP 2 aux microtubules, nous montrons que les microtubules acétylés, la MAP 2 et les filaments intermédiaires de vimentine sont disposés dans un système cytosquelettique interdépendant. Nous suggérons que ce système peut servir à stabiliser les processus dans les cellules souche neurales avant que ne soit prise la décision finale de se différencier en neurones ou en cellules gliales.

Mots clés : cellules du carcinome embryonnaire, MAP 2, microtubules, différenciation neurale, vimentine.

Introduction

The elongated processes of neural cells contain MTs aligned parallel to the long axis of the process. Up to 60% of these MTs resist depolymerization by cold or colchicine and can be described as "stable MTs" (Sahenk and Brady 1987; Brady *et al.* 1984; Black and Greene 1982). These MTs contain acetylated α-tubulin, a posttranslational modification that is associated with stable MT arrays (Piperno *et al.* 1987; Cambray-Deakin and Burgoyne 1987; Sale *et al.* 1988).

In a previous paper (Falconer *et al.* 1989), we documented the induction of this stable, acetylated MT array during neuronal differentiation in pluripotent P19 EC cell cultures (Jones-Villeneuve *et al.* 1982; Edwards and McBurney 1983). Using changes in the acetylated MT array, we classified neuronal differentiation into three stages. In the first stage, which occurs before extension of processes is seen, there is formation of a stable, acetylated MT bundle. No MAPs have been detected in association with this MT bundle. In the second stage, extension of neurites occurs and MAP 2 now colocalizes with stable MT arrays. In the third stage, further neurite specialization is seen, including onset of neurofilament, MAP 1B, and tau staining.

In this paper we examine the interactions between stable MTs and IFs. These investigations focus on vimentin, which is the first IF protein found during differentiation in EC cells (Paulin *et al.* 1982) and is also the first IF protein seen during neuronal differentiation in embryos (reviewed by Fedoroff *et al.*, 1982–1983).

Our results show that vimentin IFs are unlikely to play a role in stabilizing the early, stage 1 MT bundle. H wever, during stage 2, vimentin IFs are part f a cytoskeletal complex present in the newly extending neural process. By using two cytoskeletal disrupting agents, acrylamide, which

ABBREVIATIONS: EC, embryonal carcinoma; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; IF(s), intermediate filament(s); MT(s), microtubule(s); MAP(s), MT-associated protein(s); MEM, modified Eagle's medium; NF(s), neurofilament(s); RA, retinoic acid.

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acts to disrupt IF organizati n (Eckert 1985; Sager 1989), and estramustine, which binds to MAPs resulting in MT depolymerizati n (Stearns and Tew 1988), we demonstrate that acetylated MTs, MAP 2, and vimentin IFs are present as an interdependent complex which can serve to stabilize a newly extended process in a cell. We suggest this may occur in cells committed to the neural pathway but whose final differentiation into either neurons or glial cells is not yet specified.

Materials and methods

Cell cultures

The P19 mouse EC cell line (McBurney and Rogers 1982) was maintained at 37°C and 5% CO_2 in α-MEM (Flow Laboratories, Mississauga, Ont.), supplemented with 10% heat-inactivated FBS (Flow Labs), and passaged every 2 days using 0.25% trypsin (Flow Labs) and 1 mM EDTA (Sigma Chemical Co., St. Louis, MO) in calcium- and magnesium-free PBS. For RA (Sigma) induced neuronal differentiation, cells were seeded on coverslips, usually 1×10^5 cells per coverslip, in a 35-mm tissue culture dish in α-MEM plus 10% FBS. After 24 h, the medium was replaced with α-MEM plus 1% FBS, and 10^{-6} M RA was added to induce neuronal differentiation. A 10^{-2} M stock solution of RA was prepared in ethanol, stored at −80°C, and diluted to 10^{-4} M in medium. Appropriate amounts were added to the cultures for a final concentration of 10^{-6} M RA.

Experiments using cytoskeleton disrupting agents were performed on stage 1 (24 h after addition of RA), stage 2 (48 h after addition of RA), and stage 3 (72 h after addition of RA) cells, grown on coverslips. Colchicine (Sigma) was made up as a stock solution of 1 mg/mL in sterile distilled water, stored at −80°C, and used at a final dilution of 1 μg/mL in culture medium for 45 min. Estramustine (a gift from Mark Stearns) was freshly prepared for each experiment as a 10 mM stock solution in DMSO and used at a final dilution of 150 μM for 1 h. Acrylamide (Bio-Rad Laboratories, Richmond, CA) was freshly made up as a 4 M stock solution in sterile distilled water and used at a final dilution in culture medium of 4 mM for 5 h. When both acrylamide and colchicine were used, 1 μg/mL colchicine was added after 4 h and 15 min of exposure to 4 mM acrylamide, and the cells were fixed 45 min later.

Immunofluorescence techniques

Cells attached to coverslips were briefly washed in calcium- and magnesium-free PBS and then simultaneously fixed and extracted in 3.7% paraformaldehyde plus 0.25% glutaraldehyde (v/v) in PEM buffer (80 mM PIPES, 5 mM EGTA, 1 mM $MgCl_2$, and 1% Triton X-100 (all purchased from Sigma), pH 6.8) for 5 min, then rinsed 3 × 3 min in PBS, postfixed for 3 min in ice-cold 95% ethanol, followed by 3 × 3 min rinses in PBS. Double immunofluorescence staining was done by simultaneous incubation in two primary antibodies for 45 min at room temperature, followed by 3 × 3 min PBS rinses, and then simultaneous incubation with the two indicated secondary antibodies for 45 min at room temperature. Appropriate controls were done to insure that cross-reaction between secondary antibodies did not occur. Following 3 × 3 min PBS rinses, cells were stained for 1 min in 1 μg/mL Hoechst dye no. 33258 (Calbiochem-Behring Corp., LaJolla, CA) to visualize DNA, then rinsed 3 × 3 min in PBS. Coverslips were mounted in mounting medium containing 2.5% (w/v) of 1,4-diazabicyclo(2.2.2)octane (DABCO) (Aldrich Chem. Corp., Milwaukee, WI) dissolved in 9 parts glycerol t 1 part Tris (Bio-Rad) buffer, pH 8.6, for observation. All cells were examined with either a Zeiss Axiophot microscope or a Zeiss Universal microscope with epifluorescence optics. Micrographs were taken on Ilford XP 1-400 ASA film.

The following primary antibodies were used: 6-11B-1 mouse m noclonal antibody against acetylated α-tubulin (Piperno and Fuller 1985) was a gift from G. Pipern ; YOL 1/34, a rat m noclonal antibody which stains m st f rms of α-tubulin (Kilmartin *et al.* 1982), was purchased fr m Dimensi n Laboratories, Mississauga, Ont.; m use m noclonal antibody t MAP 2 (cl ne AP-18) (Binder *et al.* 1984) was a gift from L. I. Binder; and rabbit polyclonal anti-vimentin (Fed r ff *et al.* 1982–1983) was a gift from V. Kalnins.

The following secondary antibodies were used: FITC-conjugated goat anti-rabbit IgG (Miles-Yeda Ltd. Research Products, Elkhart, IN); rhodamine-conjugated rabbit anti-mouse IgG, cross-absorbed against rat (Zymed, Dimension Laboratories, Mississauga, Ont.); and FITC-conjugated rabbit anti-rat IgG, cross-adsorbed against mouse (Zymed).

Results

Uncommitted P19 cells were induced to differentiate along the neural pathway by addition of 10^{-6} M RA and surveyed at 24-h intervals by indirect immunofluorescence for the distribution of vimentin IFs, MAP 2, and colchicine-stable acetylated MTs. Most uncommitted P19 cells contain no filamentous vimentin, but do have a diffuse backgr und stain (Fig. 1*a*). Only a few, colchicine-stable acetylated MTs are present in uncommitted cells (Fig. 1*b*) (Falconer *et al.* 1989).

During the 24-h period after addition of RA, which we define as stage 1 differentiation, vimentin staining remains diffuse (Fig. 1*c*). However, at this time, short bundles of colchicine-stable, acetylated MTs are induced in most cells although no cytoplasmic extensions are visible (Fig. 1*d*).

During stage 2, about 48 h after RA addition, filamentous vimentin becomes a prominent feature (Fig. 1*e*). Also at this time, cytoplasmic processes are extended and are easily seen by staining the stable, acetylated MT population (Fig. 1*f*). Although differentiation is not synchronous, most processes are less than five cell bodies in length. In some cells, growth cones can be identified (arrow, Fig. 1*f*).

By stage 3, from 72 h after RA addition and onward, a complex pattern of neurons is present. When cells are double stained for vimentin (Fig. 1*g*) and acetylated MTs (Fig. 1*h*), very little colocalization of staining is observed.

Although we believed the vimention IFs in the colchicine-treated cells would collapse around the nucleus, we were surprised to find that in a small, but constant proporti n of colchicine-treated cells (less than 5% of the differentiating cells), the vimentin IF network does not collapse (Fig. 2*a*), but, instead, is similar in distribution to the acetylated, stable MT array (Fig. 2*b*). This colocalization is limited to the brief period of stage 2 differentiation. During stage 1, no filamentous vimentin is present (see Fig. 1*c*), and during Stage 3, double labeling shows very little colocalization with acetylated MTs (see Figs. 1*g* and 1*h*).

MAP 2 staining first appears during stage 2 differentiation. Double labeling of colchicine-treated cells shows that all cells with stable MTs (Fig. 3*a*) also stain for MAP 2 (Fig. 3*b*). MAP 2 can be identified in cells without processes, in cells with a single process, and in cells with two or more processes (Fig. 3*b*). However, by stage 3, double labeling of acetylated MTs (Fig. 3*c*) and MAP 2 (Fig. 3*d*) shows MAP 2 is now localized to only some of the neurites which contain stable, acetylated MTs. The MAP 2 positive processes have, presumably, differentiated into dendrites (Peng *et al.* 1986).

Since stage 2 stable, acetylated MTs colocalize with vimentin IFs and with MAP 2, vimentin IFs sh uld also colocalize

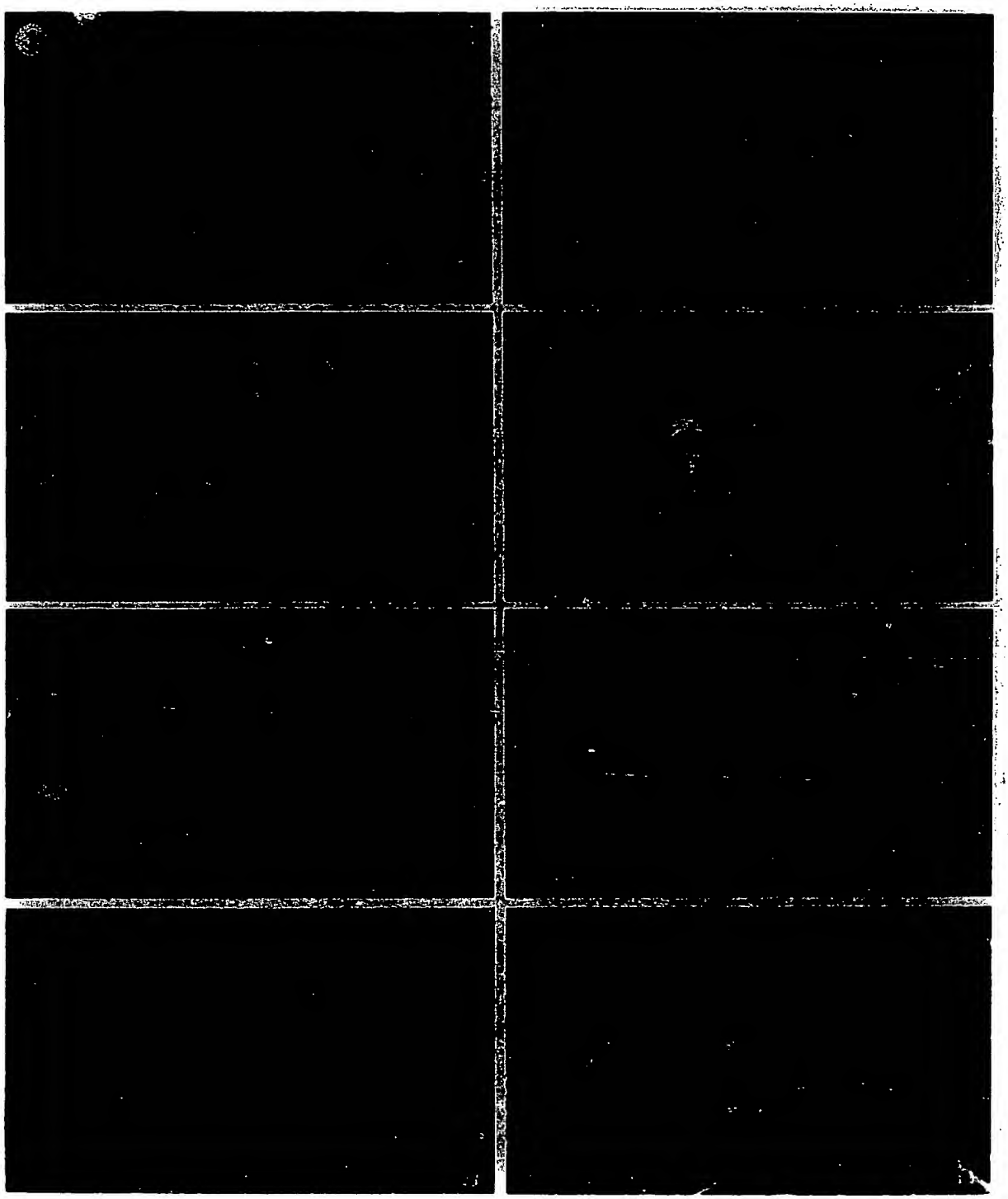

FIG. 1. P19 cells stained for vimentin (*a*,*c*,*e*, and *g*) and for acetylated MTs (*b*,*d*,*f*, and *h*) during stages of neural differentiation. Uncommitted EC cells are shown in Figs. 1*a* and 1*b*. (*a*) EC cells sh w diffuse vimentin staining. (*b*) Colchicine-treated EC cells have few stable, acetylated MTs. Differentiating stage 1 cells (24 h after RA addition) are shown in Figs. 1*c* and 1*d*). (*c*) Vimentin stain remains diffuse. (*d*) Colchicine-treated cultures now show bundles f stable, acetylated MTs. Stage 2 cells, (48 h after RA addition) are shown in Figs. 1*e* and 1*f*. (*e*) Filamentous vimentin is n w present. (*f*) In colchicine-treated cultures, the first processes containing acetylated stable MTs can be identified. Note the growth cone (arrow). Stage 3 cells, 3 days post RA, are shown, double labeled for vimentin (*g*) and for acetylated MTs (*h*). Very few processes contain vimentin (*g*), although a complex network f neurons containing acetylated MTs is evident (*h*). Magnification in *a-f*, 650×; *g* and *h*, 350×.

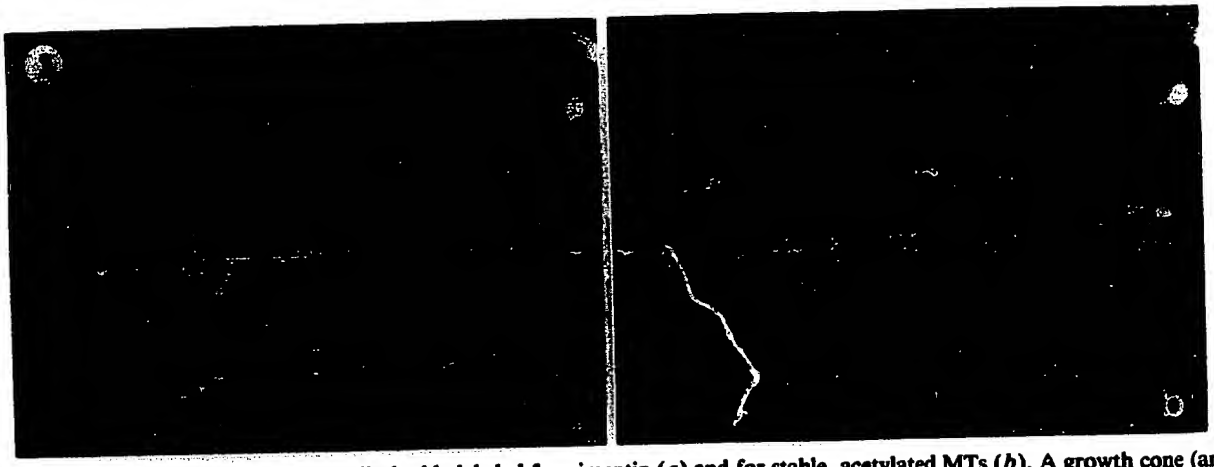

FIG. 2. A stage 2, colchicine-treated cell, double labeled for vimentin (*a*) and for stable, acetylated MTs (*b*). A growth cone (arrow) is visible. Magnification, 350×.

FIG. 3. Stage 2, colchicine-treated cells, double labeled for stable MTs (*a*) and for MAP 2 (*b*). Colocalization of MAP 2 and stable MTs can be seen in cells with no processes (arrowhead), in cells with a single process, and in cells with two processes (arrows). By stage 3, (Figs. 3*c* and 3*d*), colocalization of stable MTs (*c*) and of MAP 2 (*d*) in colchicine-treated cells, is only partial. Magnificati n in *a* and *b*, 300×; *c* and *d*, 250×.

with MAP 2. Double staining with vimentin and MAP 2 antibodies confirms this (Figs. 4*a* and 4*b*).

From these results, we postulated that during stage 2, stable, acetylated MTs, MAP 2, and vimentin IFs are linked together t form a stabilizing structure in the newly extending processes. If this linkage exists, disruption or elimination of one f the components should affect the others. To test this hypothesis, we analyzed the effect on the colchicine-stable cytoskeleton complex of two disrupting agents, 4 mM acrylamide and 150 μM estramustine. Acrylamide has been shown to induce collapse f the IF network, resulting in a "cap" of IFs near the nucleus (Eckert 1985; Sager 1989). Estramustine, a drug used in cancer therapy, binds preferentially to MAP 2, inhibiting MT assembly and thereby resulting in net depolymerizati n of MT arrays (Stearns and Tew 1988).

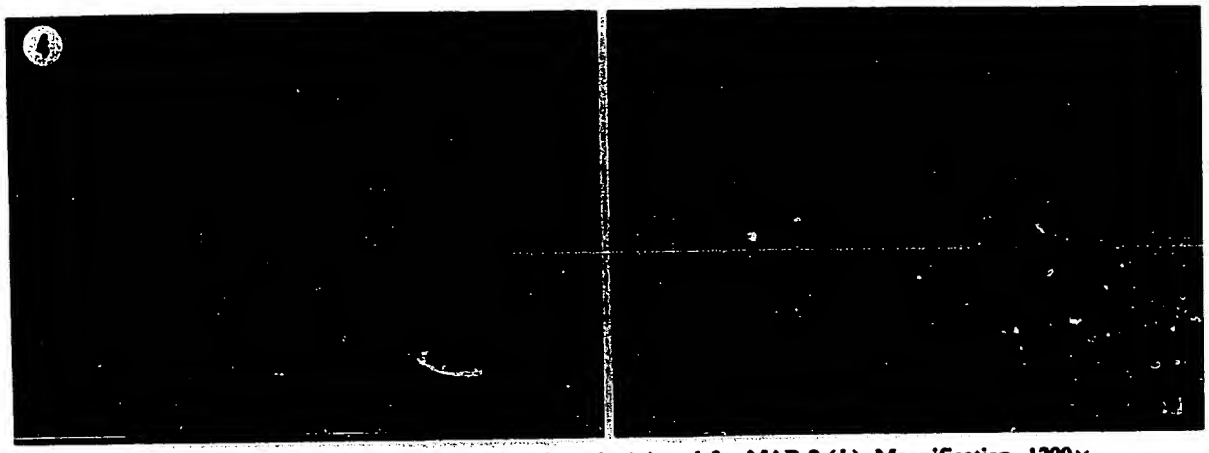

FIG. 4. Stage 2, colchicine-treated cell double labeled for vimentin (*a*) and for MAP 2 (*b*). Magnification, 1200×.

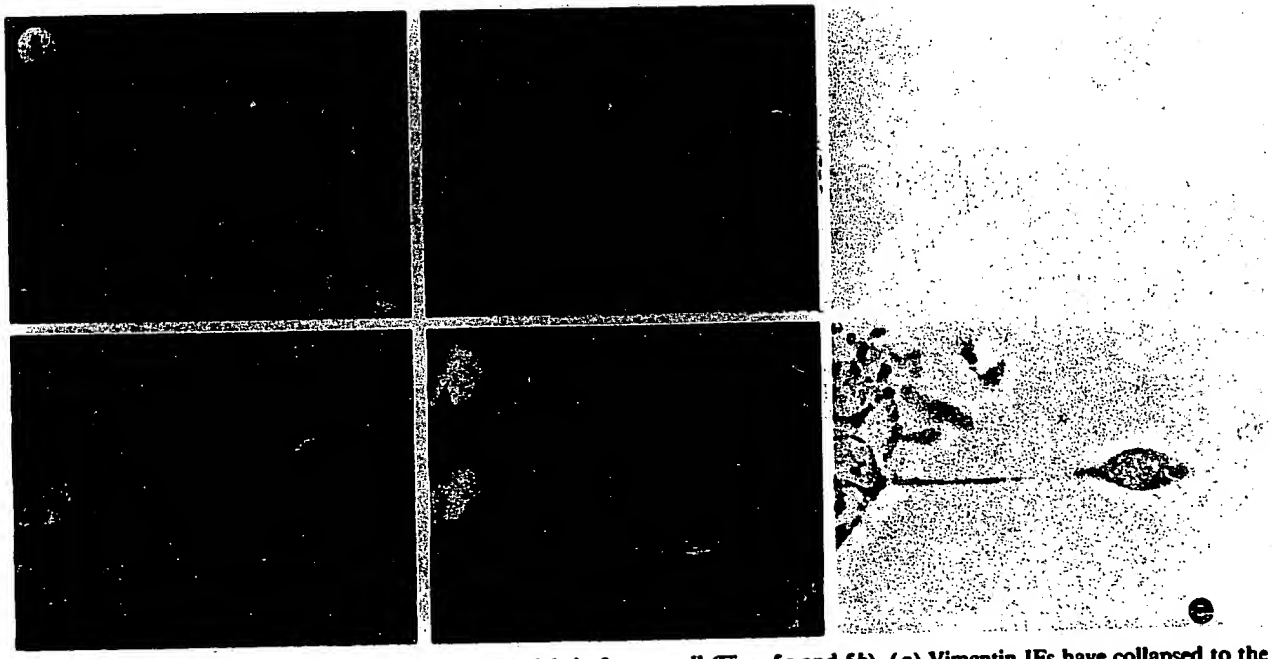

FIG. 5. Acrylamide treatment of stage 2 cells. Double label of same cell (Figs. 5*a* and 5*b*). (*a*) Vimentin IFs have collapsed to the regi n near the nucleus. (*b*) Acetylated MTs remain in the processes and cell body. To ascertain if these acetylated MTs are still stable to colchicine after collapse of the vimentin IFs, colchicine is added for the final 45 min of acrylamide treatment (Figs. 5*c*, 5*d*, and 5*e*). After this combined treatment, (*c*) vimentin IFs collapse (arrow). (*d*) No acetylated MTs remain in the process (arrow). (*e*) Phase contrast indicates the process is quite short. Magnification, 750×.

Exposure to acrylamide results in loss of vimentin IFs from the processes and formation of a diffusely staining vimentin "cap" near the nucleus (Fig. 5*a*). Acetylated MTs remain in the processes (Fig. 5*b*). MAP 2 is not detectable or is present only as a faint, diffuse cytoplasmic stain (figure n t shown). To test if these remaining acetylated MTs are still stable against colchicine-induced depolymerization, a combined drug treatment was used. Stage 2 cells were exposed to acrylamide for 5 h and colchicine was added during the last 45 min of treatment. With this procedure, vimentin IFs were collapsed and no l nger present in the process (Fig. 5*c*), no colchicine-stable acetylated MTs remained in the process (Fig. 5*d*), and MAP 2 could not be detected (figure not shown). In addition, the processes in this combined treatment were very short (Fig. 5*e*) and few in number, implying that many had completely retracted and were no longer detectable.

In estramustine-treated cells, double labeled for vimentin and acetylated tubulin, most vimentin IFs are absent from the process. The vimentin staining pattern shows a diffuse stain in the cell body and a few discrete filaments near the nucleus (Fig. 6*a*). No acetylated MTs remain in the process, and the few MTs present are colocalized to the remaining vimentin filaments (Fig. 6*b*). The process itself is quite short (Fig. 6*c*). When cells are d uble labeled for MAP 2 and vimentin, little or no MAP 2 is present in the process (Fig. 6*d*) and the vimentin IFs have collapsed and are not present in the process (Fig. 6*e*). The processes shown here (Fig. 6*f*) had not completely retracted, although in estramustine-treated cells, most processes are short and few

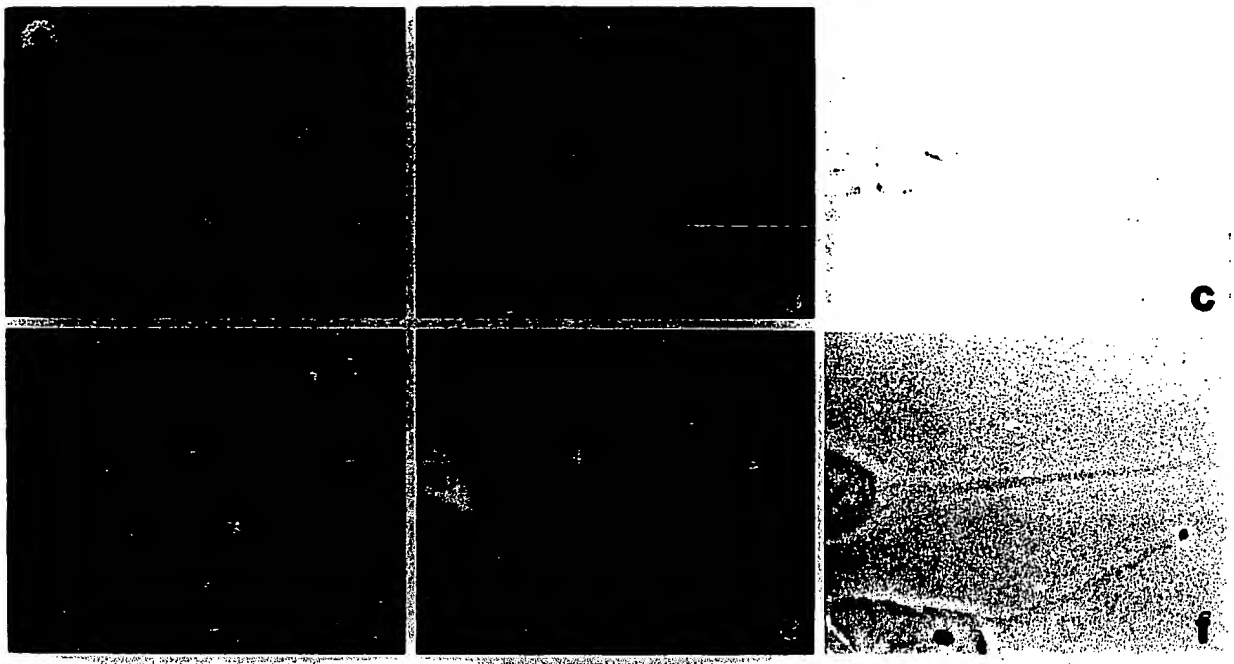

FIG. 6. Estramustine treatment of stage 2 cells. Double label of a cell for vimentin (*a*) and acetylated MTs (*b*). (*a*) Vimentin IFs collapsed near the nucleus with only a few filaments visible (arrow). (*b*) The few remaining acetylated MTs (arrow) colocalize with the vimentin IFs. (*c*) Phase contrast indicates the process is quite short. Double label of a cell for MAP 2 (*d*) and vimentin (*e*) sh ws that little MAP 2 remains in the process and the vimentin IFs collapsed near the nucleus. (*f*) Phase contrast of same cell. Magnificati n, 750×.

in number, implying that many have retracted.

Discussion

In P19 cells, during the first 24 h of neuronal differentiation (stage 1), a population of colchicine-stable, acetylated MTs appears (Falconer *et al.* 1989). It is not known what stabilizes these MTs, but stable MTs are often posttranslationally modified by acetylation of α-tubulin (L'Hernault and Rosenbaum 1983) and acetylation itself may play some role in MT stabilization (Piperno *et al.* 1987).

The initial stabilization of MTs may be enhanced at stage 2 differentiation (48 h after induction with RA) by the introduction of MAP 2, which colocalizes with colchicine-stable MTs. *In vitro* studies have shown that MAPs increase MT stability (Kirschner 1978) and influence spacing between adjacent MTs (Brown and Berlin 1985). This MAP 2 mediated stabilization of differentiating cells is apparently also a transient phenomenon as shown by the results in this article.

In P19 cultures induced to differentiate along neural pathways, the simultaneous colocalization of stable acetylated MTs, MAP 2, and vimentin IFs is seen only during the brief period of stage 2, when process outgrowth first appears. When this cytoskeletal complex is treated with disrupting agents, perturbation of one element of the complex results in reorganization or altered stability of the other elements. This suggests that the three elements are associated together with MAP 2 as the probable linking agent between the MTs and vimentin IFs. MAP 2 has been suggested to be a cross-linking protein between MTs and IFs (reviewed by Matus, 1988), and an association f MAP 2 with MTs and vimentin in flat cells from primary cultures f mature rat brain has been demonstrated (Bloom and Vallee 1983).

In differentiated cells *in situ*, vimentin is characteristic of cells of mesenchymal origin, but is also expressed by and considered a marker of cells derived from the neuroectoderm, including astrocytes and radial glial cells (Schnitzer *et al.* 1981). Most continuous cell lines express vimentin; however, filamentous vimentin staining in P19 cells is almost certainly related to differentiation and is not a result f culturing the cells *in vitro*. In uncommitted EC cultures, we find only about 15% of cells have some amount of filamentous vimentin staining. The remaining cells have a diffuse background stain, which may indicate the presence of unpolymerized vimentin. Significant amounts of filamentous vimentin are not seen in our cells until 48 h after the addition of RA. Paulin *et al.* (1982) documented the occurrence of filamentous vimentin in 17 different EC cell lines (not including the P19 line) and found that the percentage of cells containing vimentin varies from line to line, but all lines express more vimentin after treatment with RA.

During differentiation, vimentin is the first IF pr tein expressed in P19 cells (Levine and Flynn 1986). NF staining is not seen until 3 or more days after addition of RA (Levine and Flynn 1986; Vielkind *et al.* 1989), while GFAP arises at least several days after NFs are seen (Jones-Villeneuve *et al.* 1982). Since it is NF and GFA proteins that are considered the definitive markers for neurons and glial cells, respectively, it is not possible to predict what will be the final differentiated state of stage 2 cells.

During development of the mouse embryo nervous system, vimentin is first detected on the 9th day of gestation (E9), in processes arising from the basal plate neuroepithelium of the neural tube (Houle and Fedoroff 1983). However, neither NFs nor GFAP are expressed at this stage f mouse embry development (Schnitzer *et al.* 1981; Bignami *et al.* 1982). Houle and Fedoroff (1983) and Schnitzer *et al.* (1981) suggest that the first cells to express

vimentin may be ventricular cells, a neural stem cell which retains the capacity for multipotential differentiation along either neuronal or glial pathways.

The absence of vimentin IFs from P19 cells during the first 24 h of neural differentiati n may be analogous t the absence of vimentin in the neural tube before embryonic day 9 (E9). Similarly, the period of colocalization of vimentin IFs with acetylated MTs and with MAP 2 in newly extending processes of P19 cells may correspond to the time when filamentous vimentin appears in processes extended by E9 ventricular cells.

The vimentin IF - MAP 2 - acetylated MT complex may be present in a relatively unspecified stem cell which retains the ability to modify various components during subsequent differentiation. Thus, we suggest that acetylated MTs, found both in neurites (Cambray-Deakin and Burgoyne 1987) and in glial processes (Sale *et al.* 1988), will remain, regardless f the subsequent differentiation pathway. If the cell commits to the glial lineage, acetylated MTs and vimentin IFs will remain and GFAP will be subsequently added. However, MAP 2 may be replaced by MAP 1B or another MAP. If the neuronal lineage is chosen, vimentin IFs first will coexist with NF protein and then will be replaced by NF protein, as seen during neuronal differentiation in embryos (Ziller *et al.* 1983; Bignami *et al.* 1982). MAP 2 will remain in dendritic processes and will be replaced by another MAP in axons.

In differentiating P19 cells, and possibly during embryogenesis as well, the stabilization of a newly extended process may itself be important in determining the final differentiation path, particularly if surrounding cells providc a signal that is perceived by the extension.

Our results also substantiate that the P19 EC cell culture is a good model system to study neurogenesis. Unlike neuroblast ma and pheochromocytoma (PC 12) cultures, which derive from neurally committed cells, the P19 culture consists of pluripotent cells (Jones-Villeneuve *et al.* 1982; Edwards and McBurney 1983). Thus, early events, which may have already taken place in neuroblastoma and PC 12 stem cells, can be observed in differentiating P19 cells. In addition, differentiating neurons in P19 cultures have the same alterations in IF population and distribution as reported *in vivo* (Levine and Flynn 1986). However, neurogenesis in neuroblastoma and PC 12 cultures is atypical, with neurons retaining both vimentin IFs and neurofilaments throughout differentiation (Shea and Nixon 1988).

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